



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : <b>C12N 15/00, 15/05, 15/29, 15/64, 15/82, A01H 1/00, 4/00</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 97/13851</b> (43) International Publication Date: <b>17 April 1997 (17.04.97)</b></p>
<p>(21) International Application Number: <b>PCT/US96/16387</b> (22) International Filing Date: <b>11 October 1996 (11.10.96)</b> (30) Priority Data: <b>60/005,404</b>      <b>13 October 1995 (13.10.95)</b>      <b>US</b> (71) Applicant (for all designated States except US): <b>PURDUE RESEARCH FOUNDATION [US/US]; Office of Technology Transfer, Room 328 ENAD, Purdue University, West Lafayette, IN 47907 (US).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>HANDA, Avtar, K. [IN/US]; 3420 Boone Street, West Lafayette, IN 47904 (US). KAUSCH, Kurt, D. [US/US]; Apartment 2, 1457 W. Walton, Chicago, IL 60622 (US).</b> (74) Agents: <b>HENRY, Thomas, Q. et al.; Woodard, Emhardt, Naughton, Moriarty &amp; McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204-5137 (US).</b></p>		<p>(81) Designated States: <b>AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i></p>
<p>(54) Title: <b>IMPROVEMENT OF FRUIT QUALITY BY INHIBITING PRODUCTION OF LIPOXYGENASE IN FRUITS</b> (57) Abstract  The present invention relates generally to a transgenic fruit-bearing plant having a foreign nucleotide sequence inserted therein which is substantially similar to a portion of the plant's fruit ripening specific lipoxygenase cDNA. Transgenic plants according to the present invention produce fruits having modified and surprisingly superior ripening characteristics, including improved quality and texture, greater firmness, longer shelf life, better packaging and storage characteristics and improved processing characteristics. Also provided are transgenic fruits; transgenic plant cells; methods for making inventive transgenic plants, fruits and plant cells; methods for inhibiting lipoxygenase production in plants; isolated nucleotide sequences; and vectors comprising these isolated nucleotide sequences.</p>		

\* (Referred to in PCT Gazette No. 40/1997, Section II)

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

# IMPROVEMENT OF FRUIT QUALITY BY INHIBITING PRODUCTION OF LIPOXYGENASE IN FRUITS

## BACKGROUND OF THE INVENTION

### Field of the Invention

5       The present invention relates to isolated nucleotide sequences; methods for using the sequences to make transgenic plants which produce edible fruits having improved firmness and longevity; and vectors having the nucleotide sequences incorporated therein. More  
10 specifically, the present invention relates to transgenic plants and methods for making the same, the genomes of said plants having incorporated therein foreign nucleotide sequences which function to inhibit production of fruit ripening specific lipoxygenase ("FRS-LOX") in a ripening  
15 fruit. The inhibition of FRS-LOX gene expression provides a mechanism to improve characteristics of a fruit, including controlling fruit senescence and tissue softening associated with post-maturation processes.

### Discussion of Related Art

#### 20 Biochemistry of Lipoxygenases

Lipoxygenases ("LOXs") are nonheme iron-containing dioxygenases that catalyze the incorporation of molecular oxygen into unsaturated fatty acids containing cis, cis 1,4-pentadiene structure to yield a 1-hydroperoxy-2-4-trans, cis  
25 pentadiene product. Typical substrates for LOXs in plants are linoleate (C18:2) and linolenate (C18:3) fatty acids, while animal LOXs prefer arachidonate (C20:4). Some LOXs are able to act on substituted fatty acid substrates, while

- 2 -

others require free fatty acids. LOXs can vary in respect to (1) the site of primary hydrogen abstraction, (2) the direction of the double bond shifts in the primary radical leading to the hydroperoxide product and (3) the stereospecificity of both hydrogen abstraction and dioxygen insertion (Ford-Hutchinson et al., Annu. Rev. Biochem. 63:383, 1994).

In plants, most fatty acids are esterified to a glycerol backbone in the form of glycerolipids and lipases are thought to cleave these phospholipids into usable plant LOX substrates (free fatty acids). The LOX-catalyzed fatty acid hydroperoxides serve as intermediates for a number of secondary reactions leading to jasmonic acid, traumatin, traumatic acid, volatile alcohols (hexanal), aldehydes, ketols and 9C oxo fatty acids (Vick and Zimmerman, Biochemistry of Plants, Vol. 9:53, 1987; Hildebrand, Physiol. Plantarum 76:249, 1989). The 5-LOX from humans is representative of a unique type of LOX that requires the association of the 5-LOX Activating Protein (FLAP) for activity. Also, a LOX from the rabbit reticulocyte has been reported to attack mitochondrial membranes in the absence of any lipid hydrolyzing enzymes during the maturation of erythroid cells (Schewe et al., Adv. Enzymol. 58:191, 1986).

#### Lipoxygenases: A Multigenic Family

LOXs have been found in a wide range of organisms including higher plants, animals, yeast, fungi and cyanobacterium (Siedow, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:145, 1991). LOX multigenic families have been characterized in several plant and animal species (Siedow, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:145, 1991; Ford-Hutchinson et al., Annu. Rev. Biochem. 63:383, 1994). The best characterized plant LOXs are the three soybean cotyledon LOX monomer isozymes; L-1, L-2 and L-3, all globular, water soluble proteins with MWs of about 96 kD. LOXs from rice, soybean, cotton, sunflower, tomato,

- 3 -

Arabidopsis, cucumber, kiwi and tobacco are some that have been identified and are on the order of 95kD, with the exception of pea (72-108 kD). Sequences reported for plant LOXs are approximately 60% homologous to one another. Human LOXs are about 60% homologous and are only 25% identical to plant LOXs (Ford-Hutchinson et al., Annu. Rev. Biochem. 63:383, 1994). Some of the plant LOXs are larger than animal LOXs and show homology with them only in limited regions (Minor et al., Biochem. 32:6320, 1993).

#### 10 Biological Role(s) of Lipoxygenases

The function of various LOX isozymes in plant and mammalian systems is unknown. The hydroperoxide products from some animal LOXs serve as precursors in the production of leukotrienes and lipoxins, regulatory molecules involved in responses include leukotiene induced altered cell functions such as chemotaxis and chemokinesis. Roles of LOX during all stages of plant growth and development have been speculated (Siedow, Annu. Rev. Plant Physiol. Plant Mol. Bio. 42:145, 1991). LOX activity has been demonstrated in rapidly growing young tissues (germinating seedlings). It has been suggested that jasmonic acid and hydroperoxide free radicals, primary and secondary products of LOX, may play roles in plant senescence by promoting cell membrane deterioration, inhibiting protein synthesis and chloroplast photochemical activity. Upon tissue wounding, increases in LOX activity and mRNA accumulation have been seen in some plants. Traumatins and traumatinic acid may be involved in wound healing of damaged plant tissues (Hildebrand, Physio. Plantarum 76:249, 1989). Another secondary LOX product, hexanal, may be produced in response to pest/pathogen attack. However, no evidence to support any of these hypothesis has been obtained.

- 4 -

Shelf Life of Fruits and Vegetables

Fruits and vegetables are highly perishable crops and significant losses often occur after their harvest but before they reach the consumer. The primary causes of these losses are the inability to control: 1) senescence of these crops; 2) the ripening process in fruits; and 3) ripening-and senescence-associated tissue softening. One of the objectives of plant breeders for many years has been to control these processes by introducing traits from wild germ plasm into the cultivated species. In recent years, attempts have been made to use recombinant DNA technology to modify some of these traits. Both antisense and co-suppression technologies have been used in some crop plants to modify the expression of specific genes which may have deleterious effects on plant growth and development or crop productivity in general. However, none have proven fully satisfactory in reducing the rate of tissue softening associated with post-maturation plant processes.

Major transitions in fruit development and metabolism accompany the initiation of fruit ripening. In addition to alterations in pigment biosynthesis and production of volatile compounds, fruits undergo significant changes in texture during ripening. The biochemical bases for ripening- and senescence-associated fruit softening are not yet understood; however, dissolution of the middle lamella and cell wall separation due to depolymerization of pectins by polygalacturonase as well as loss of calcium have been suggested to contribute to fruit softening. Only slight improvement in fruit integrity has been reported in fruits with low polygalacturonase activity (Schuch et al. HortScience 26: 1517, 1991; Carrington et al., Plant Physiology 103: 429, 1993).

There is a need for transgenic plants which produce fruits having modified ripening and post-maturation characteristics including improved quality and texture, greater firmness, longer shelf life, better packing and

- 5 -

storage characteristics and improved processing characteristics.

- 6 -

## SUMMARY OF THE INVENTION

The invention described herein features inhibiting the expression of fruit ripening specific lipoxygenase ("FRS-LOX") in a plant, specifically in cells of a fruit of the plant. For instance, foreign DNA can be introduced into cells to reduce FRS-LOX production. In a preferred embodiment, the cell is a plant cell and the genetic material is a sense or antisense fragment substantially similar to a portion of the FRS-LOX cDNA shown in SEQ ID NO:4. Most preferably, the cell is a cell of a fruit-bearing plant, such as a tomato plant cell.

It is expected that the present invention can be applied to the inhibition of FRS-LOX gene products in a wide variety of useful plants. These may include, for example, commercially important fruit-bearing plants in which post-maturation weakening reduces economic value, such as melons, peaches, bananas, apples, strawberries, kiwi fruit, and in particular the tomato.

The present inventors have made the surprising discoveries that (1) inhibition of FRS-LOX greatly improves fruit qualities such as, for example, firmness and shelf life; and (2) antisense and co-suppression (sense) technologies can be successfully used to inhibit FRS-LOX gene expression in plants so as to provide fruits having superior qualities such as, for example, firmness and shelf life. It is believed that improved fruit qualities result from reduced activity of degradative pathways (e.g., membrane deterioration); however, it is not intended that the present invention be limited to this theory.

Briefly describing one aspect of the present invention, there is provided a method for making a transformed plant having improved fruit quality comprising inserting a nucleotide sequence into DNA of the plant in a sense or antisense orientation under a suitable promoter so as to inhibit production of lipoxygenase in the fruit of the plant as it ripens. In a preferred method, a sequence of



- 7 -

nucleotides is inserted into a target cell by providing a vector comprising the nucleotide sequence and contacting the vector with the target cell.

5 Additional aspects of the present invention include vectors having incorporated therein nucleotide sequences having substantial similarity to all or a portion of the sequence of SEQ ID NO:4 (FIG. 4).

10 Additional aspects of the invention include constructs selected from the group consisting of pMLSL, pMLAL, pUSL2, pUAL2, pUEL300S and pUEL300A, which are useful for inserting foreign DNA into a plant cell genome.

15 According to other aspects of the present invention there are provided transgenic fruits, transgenic plants and transgenic host cells, preferably plant cells such as germ cells and cotyledon cells. These various transgenic hosts are preferably transformed by having incorporated into their genomes, nucleotide sequences as delineated above and described in greater detail below.

20 It is an object of the present invention to provide transgenic plants which produce fruits having modified ripening characteristics, including improved quality and texture, greater firmness, longer shelf life, better packaging and storage characteristics and improved processing characteristics.

25 Further objects, advantages, and features of the present invention will be apparent from the detailed description which follows.

- 8 -

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 sets forth the nucleotide sequence of one portion of the fruit ripening specific lipoxygenase ("FRS-LOX") gene which is inserted into a plant cell genome according to one preferred embodiment of the present invention.

FIG. 2 sets forth the nucleotide sequence of another portion of the FRS-LOX gene which is inserted into a plant cell genome according to another preferred embodiment of the present invention.

FIG. 3 sets forth the nucleotide sequence of another portion of the FRS-LOX gene which is inserted into a plant cell genome according to another preferred embodiment of the present invention.

FIG. 4 sets forth the nucleotide sequence of the coding region of a tomato FRS-LOX gene cloned and characterized for use in accordance with the present invention.

FIG. 5A shows various regions of the tomato FRS-LOX cDNA advantageously used for making vectors according to the present invention.

FIG. 5B shows the 2871 base pair region of the tomato FRS-LOX cDNA shown in FIG. 5A, which is preferably used for making pUSL2 and pUAL2 according to the present invention.

FIG. 5C shows the 2440 base pair region of the tomato FRS-LOX cDNA shown in FIG. 5A, which is preferably used for making pMLSL and pMLAL according to the present invention.

FIG. 5D shows the 297 base pair region of the tomato FRS-LOX cDNA shown in FIG. 5A, which is preferably used for making pUEL300A according to the present invention.

FIG. 6 is a plot of fruit firmness vs. the number of days after breaker for a transgenic tomato of the present invention (650-1) and an Ohio control tomato. "Breaker," as used herein, is intended to denote the time at which a tomato begins to change color from green to orange.

SUBSTITUTE SHEET (RULE 26)

- 9 -

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, references will now be made to certain embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended. Any alterations and modifications in the invention, and such further applications of the principles of the invention therein being contemplated as would normally occur to one skilled in the art to which the invention relates.

In accordance with the present invention, there are provided transgenic plants and fruits and methods and materials for making them. It has been discovered that the expression of the fruit ripening specific lipoxygenase ("FRS-LOX") gene, and correspondingly, the level of FRS-LOX enzyme activity, are implicated in causing fruit weakening and membrane deterioration in post-maturation fruits. It has also been discovered that these processes can be suppressed in plants by the introduction of a foreign nucleotide sequence having substantial similarity to all or a portion of the coding sequence of the FRS-LOX gene (e.g., as set forth in FIG. 4, SEQ ID NO:4) in either the sense or the antisense orientation. The invention thus provides recombinant DNA with which to achieve such suppression, methods for transforming plants to achieve such suppression, and the resultant transgenic plant cells, transgenic plants and transgenic fruits thereof. The term "substantial similarity," as used herein, is intended to mean sufficiently similar to cause improved fruit quality by inhibiting lipoxygenase production in a fruit when inserted in sense or antisense orientation. For example, it is contemplated that nucleotide sequences useful in the invention will hybridize, under stringent hybridization conditions, to the coding sequence of the FRS-LOX gene (e.g., the nucleotide sequence of SEQ ID NO:4; FIG. 4).

- 10 -

The Examples given below clearly show that the methods of the invention, using the expression of a nucleotide sequence comprising a portion of the FRS-LOX cDNA, results in substantial inhibition of FRS-LOX. Tomato fruit and their seeds, for example, and progeny of these plants will find use in the production of new tomato varieties containing reduced FRS-LOX. These plants are useful in the production of tomatoes of improved quality, which have a longer storage life, better transportability, better field holding (i.e., fruit lasts longer in good condition on the plant prior to harvest) or be easier to process, and may produce improved products such as whole peeled tomatoes, puree, ketchup or sauces. It will be understood that an FRS-LOX gene appears not only in tomatoes, but also in a wide variety of other plants and that the invention can be used not only for the inhibition of tomato FRS-LOX, but also for the inhibition of FRS-LOX and similar enzymes in various other fruit-bearing plant species. In these other species, of course, the inserted foreign nucleotide sequence must have substantial similarity to all or a portion of the FRS-LOX gene associated with that species.

The preferred aspects of the present invention are carried out using the FRS-LOX gene. The cDNA clone for a tomato FRS-LOX gene (shown in FIG. 4; SEQ ID NO:4) has 2871 nucleotide base pairs and an open reading frame encoding a protein of 859 amino acids with calculated molecular mass of 97kD and pI of 5.5. Comparison of its sequence reveals 73 and 82 percent similarity at nucleic acid and amino acid levels, respectively, to a LOX cloned from wounded potato tubers which show 5-LOX activity on arachidonic acid (Mulliez et al., Biochem. Biophys. Acta 916,13, 1987; Casey, Plant Physiology 107:265, 1995).

In accordance with the present invention, a nucleotide sequence having substantial similarity to all or a portion of the coding sequence of the FRS-LOX gene (SEQ ID NO:4) is incorporated in a recombinant DNA molecule under the control

- 11 -

of a promoter. In this regard, a recombinant DNA molecule is one which has either been naturally or artificially produced from parts derived from heterologous sources, which parts may be naturally occurring or chemically synthesized molecules, and wherein those parts have been joined by ligation or other means known in the art. The introduced FRS-LOX coding sequence is under control of the promoter and thus will be generally downstream from the promoter. Stated alternatively, the promoter sequence will be generally upstream (i.e., at the 5' end) of the coding sequence.

A constitutive promoter was used in the methods described in the Examples below. However, targeting of the gene product can be obtained using a constitutive (e.g. Cauliflower Mosaic Virus 35S promoter), inducible (e.g. Tomato E8 ethylene inducible promoter) or developmentally regulated (e.g. Tomato polygalacturonase promoter) promoter to construct the vectors.

With respect to the function of the promoter, it is well known that there may or may not be other regulatory elements (e.g., enhancer sequences) which cooperate with the promoter and a transcriptional start codon to achieve transcription of the introduced (i.e., foreign) sequence. The phrase "under control of" contemplates the presence of such other elements as are necessary to achieve transcription of the introduced sequence. Such transcription can be assessed, for example, by the detection of the mRNA products of the same. Also, the recombinant DNA will preferably include a termination sequence downstream from the introduced sequence.

The introduced sequence according to the instant invention is preferably a nucleotide sequence having substantial similarity to all or a portion of the nucleotide sequence that encodes the FRS-LOX enzyme (see for example, SEQ ID NO:4). As used herein, the term "portion" is intended to refer to a nucleotide sequence having a sufficient number of nucleotides to cause improved fruit

- 12 -

quality by inhibiting lipoxygenase production in a fruit when inserted in sense or antisense orientation. In this regard, coding sequences of about 90 base pairs have been shown to possess inhibitory properties in sense  
5 interactions. (See Vaueheret H. (1993) Identification of a General Silencer for 19S and 35S Promoter in a Transgenic Tobacco Plant: 90 bp of homology in the promoter sequence are sufficient for transactivation. C.R.Acad.Sci. III 316:1471-1483.)

10 According to the present invention, the introduced sequence can have either a sense or an antisense orientation, and can contain nucleotide sequences from any suitable source, including both natural and synthetic sources. In a preferred embodiment, the introduced sequence  
15 is one of those of FIGS. 1-3 (SEQ ID NOS:1-3) or one having substantial similarity to one of these sequences, and is incorporated in either a sense or an antisense orientation.

One preferred vector according to the present invention, for example the pMLSL construct, comprises the  
20 nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) in the sense orientation (See Example 3, below). The DNA insert shown in SEQ ID NO:1 is a 2440 base pair DNA fragment representing nucleotides 158 to 2598 of the fruit FRS-LOX gene shown in FIG. 4 (SEQ ID NO:4). Another preferred  
25 vector, for example the pMLAL construct (See Example 3, below), comprises the same DNA fragment inserted into the vector in the antisense orientation (reverse order).

Another nucleotide sequence which is advantageously inserted into a vector according to the present invention is  
30 a sequence containing the full-length FRS-LOX cDNA (SEQ ID NO:4) having 2871 base pairs. This nucleotide sequence is shown in FIG. 2 (SEQ ID NO:2). This sequence may also be inserted in either the sense orientation (See, for example, the pUSL2 construct described in Example 4, below) or the  
35 antisense orientation (See, for example, the pUAL2 construct construct described in Example 4, below).

- 13 -

A third nucleotide sequence which is inserted into a vector according to a preferred embodiment of the present invention is a sequence containing nucleotides 1 through 297 of the FRS-LOX gene shown in FIG. 4 (SEQ ID NO:4). This sequence is also set forth in FIG. 3 (SEQ ID NO:3). As with the previously-described sequences, this nucleotide sequence may be inserted into a vector in either the sense orientation (see, for example, the pUEL300S construct described in Example 5, below) or the antisense orientation (see, for example, the pUEL300A construct described in Example 5, below). These vectors are all useful for making inventive transgenic plants as described above.

Additionally contemplated by the present invention is a vector having incorporated therein a nucleotide sequence substantially similar to one of the above-described nucleotide sequences.

Suitable nucleotide sequences for use as starting materials in the present invention can be isolated from DNA libraries obtained from other species by means of nucleic acid hybridization or PCR, using as hybridization probes or primers for FRS-LOX, nucleotide sequences that have been published for FRS-LOX genes. Alternatively, antibodies to the FRS-LOX protein can be used to screen a plant cDNA library for clones that express the FRS-LOX protein. The cDNA thus identified as FRS-LOX-protein-encoding can then be used to isolate a genomic clone containing a similar nucleotide sequence or a portion thereof. For an illustrative list of clones obtained in this way, see Table 1 in Example I below.

In accordance with the present invention, the FRS-LOX insertion sequence can be, but is not necessarily, a mutant form. Mutations may include, for example, insertions, deletions, and/or substitutions of one or more nucleotides. Such a mutation in accordance with the invention will provide a coding sequence which, when inserted into a plant in the sense or antisense orientation under the control of

- 14 -

a promoter that is expressed in the plant, achieves the suppression of the expression of a natural FRS-LOX gene and the FRS-LOX activity of the transgenic plant.

Recombinant DNA in accordance with the invention can be incorporated into vectors and introduced into the genome of plants using conventional techniques. In this regard, the term "genome" as used herein is intended to refer to DNA which is present in the plant and which is heritable by progeny during propagation of the plant. For example, the invention is illustrated in the Examples below utilizing *Agrobacterium tumefaciens*-mediated transformation, although other techniques can also be used and are within the purview of the ordinarily skilled artisan. The technique used for a given plant species or specific type of plant tissue will depend upon the known successful techniques. Additional means for introducing recombinant DNA into plant tissue include but are not limited to electroporation, microprojectiles, microinjection, as well as other T-DNA mediated transfer from *Agrobacterium tumefaciens*.

Once the recombinant DNA is introduced into the plant tissue, successful transformants can be screened using standard techniques such as the use of marker genes, e.g., genes encoding resistance to antibiotics. Additionally, the level of expression of the natural FRS-LOX gene of transgenic plants may be measured at the transcriptional level or as protein synthesized.

Transgenic plants in accordance with the present invention can also be identified by detection of a significant increase in the firmness of the fruit of the transgenic plant as compared to its wild-type counterparts. The level of firmness of a whole fruit can be determined, for example, by using conventional testing equipment such as a McCormick Fruit Tech (Yakima, WA) firmness pressure tester. This tester has a plunger tip and measures the penetration force necessary for the plunger tip to enter the fruit epidermis. In performing these tests, about a 1 cm square



- 15 -

section of the waxy epidermis from each fruit was removed where the measurement was to be taken. A minimum of six fruits were analyzed and the mean and standard error were calculated. The results of various firmness tests are given in Table 3 below.

Fruits produced by transgenic plants made according to the present invention are superior to other fruits due to the advantageously modified ripening characteristics including improved quality and texture and greater firmness. These characteristics are believed to result from reduced activity of degradative pathways in the fruit (e.g., membrane deterioration); however, the present invention is not intended to be limited by this theory. These characteristics are economically important, for example, because they impart to the fruit improved shelf life, better packaging and storage characteristics and improved processing characteristics. The present invention provides fruits which are much more easily handled than other fruits, and which are much less likely to be bruised or smashed in the normal course of harvesting, handling, transportation and delivery to a consumer.

According to the present invention, a partial inhibition of lipoxygenase production is adequate for improving fruit quality. Varying levels of inhibition can be obtained by either selecting other transformants, using different fragments of the nucleotide sequence of the FRS-LOX gene (e.g., SEQ ID NO:4) or using a different promoter, as will be apparent by those skilled in the art. In this regard, preferred transgenics of the invention exhibit at least about a 50% reduction in the level of natural FRS-LOX gene expression or FRS-LOX activity, and in more preferred transgenic plants, reduction is 60% or more. Most preferably, transgenic plants of the invention exhibit at least about 70% reduction of these processes.

Transgenic plants in accordance with the invention can be cultured and reproduced under standard conditions and

- 16 -

using standard techniques. Likewise, transgenic fruits obtained from transgenic plants can be conventionally used and processed.

The invention will be further described with reference to the following specific Examples. However, it will be understood that the Examples are offered to further illustrate the present invention, but are not to be construed as limiting the scope thereof.

#### EXAMPLE ONE

##### Molecular Cloning of FRS-LOX cDNA

A 90 kd protein that accumulates in the red-ripe fruit of a tomato plant, purified by ammonium sulfate precipitations, ion-exchange chromatography and SDS-PAGE was used to produce polyclonal antibodies in a rabbit. These antibodies were used to immunoscreen more than 150,000 recombinant clones from an expression cDNA library made from poly A+RNA from red-ripe tomato pericarp in the UNI-ZAP XR vector. Among the 24 cDNA clones obtained, 17 clones were similar and had inserts which showed 60-65% homology with the LOX gene family (Gen Bank and EMBL sequence libraries). One of the cDNAs obtained was used to rescreen the cDNA library to obtain a full-length FRS-LOX gene (Kausch and Handa, Plant Physiology 107:669, 1995). FIG. 4 shows the nucleic acid sequence of this gene. Table 1 shows percent similarities of this FRS-LOX gene to LOX clones from other plant species.

- 17 -

**Table 1. Comparison of the fruit ripening specific lipoxygenase gene sequences at DNA and amino acid levels with other plant Lipoxygenases.**

5	Lox Gene Accession #	Percent Similarity	
		Nucleic Acid	Amino Acid
	Leu09025	99.5	99.1
	Leu09026	72.7	81.7
	Stlox1	73.2	81.6
10	Athlipoxy	64.6	75.7
	Athatlo	51.6	61.4
	Pvlipoxy	64.8	74.9
	Soylox	61.4	69.7
	Soyloxb	62.8	73.3
15	Soyloxc	62.7	67.6
	Pslipox	61.4	71.9
	Gmu04526	62.0	73.0
	Gmu04785	54.1	71.2
	Pslipocy	61.7	73.7
20	Oslma	54.1	71.2
	Ric120p	48.2	61.2

- 18 -

**EXAMPLE TWO****Construction of Vectors**

Several sense and antisense vectors containing different regions of the cloned FRS-LOX (FIG. 4) were made to create transgenic tomato plants. Table 2 summarizes the vectors made and specific procedures used to prepare them are described in Examples 3-6.

Table 2. Summary of Sense and Antisense LOX Vectors.

10	Name of Sense Vector	Name of Antisense Vector	Insert Size
	pMLSL	pMLAL	2440 bp
	pUSL2	pUAL2	2871 bp
	pUEL300S	pUEL	300A 297 bp

**EXAMPLE THREE****Construction of pMLSL/pMLAL**

1. A 1.7 kb *EcoRI* and *ClaI* DNA fragment containing the Cauliflower Mosaic Virus (CaMV 35S) promoter and the small subunit of the Ribulose 1,2-Bisphosphate Carboxylase/Oxygenase (*rbcS*) 3' terminator with intervening multi-cloning sites was isolated from pKYLX7 (Schardl et al., Gene 61:1, 1987) and cloned into pTZ18U to generate vector pTZ35rbcS.

2. A 2440 bp DNA fragment representing nucleotides 158 to 2598 of the fruit FRS-LOX (FIG. 1, SEQ ID NO:1) was isolated from a partial LOX clone 8-27-1 and ligated into the multi-cloning sites present between the CaMV 35S promoter and *rbcS* 3' terminator sequences in pTZ35rbcS in both orientations. The clone with the LOX cDNA in the sense orientation relative to the CaMV 35S promoter was named pTZSL, while the clone with the LOX cDNA in the antisense orientation relative to the CaMV 35S promoter designation as

- 19 -

pTZAL. Restriction mapping and Southern blotting with CaMV 35S, LOX and rbcS 3' probes and DNA sequencing were used to establish structure of pTZSL and pTZAL.

- 5 3. The 4.1 kb DNA fragments containing the CaMV 35S promoter, 2440 bp LOX DNA fragment in sense or antisense orientation and rbcS 3' terminator were obtained from pTZSL or pTZAL, respectively. These fragments were cloned between the *Eco RI* and *Sma I* sites in pMLJ1 (an *Agrobacterium*  
10 based transformation vector) both in sense and antisense configuration to obtain pMLSL and pMLAL, respectively. Restriction mapping and Southern blotting with CaMV 35S, LOX and rbcS 3' probes and DNA sequencing were used to establish structures of pMLSL and pMLAL.

15

#### EXAMPLE FOUR

##### Construction of pUSL2/pUAL2

1. A 0.6 kb DNA fragment (0.6 kb) containing the rbcS terminator was isolated from pTZ35rbcS and cloned at Xba I site of pGEM112 to create pG11rbcX.  
20
2. The CaMV35S promoter (1.1 kb) was isolated from pTZ35rbcS after digestion with *Sac I* and *Hind III*, ligated with *Hind III-Eco RI* linkers, and forced cloned between *Sac I* and *Eco RI* sites of pG11rbcX to create pG35rbcX.  
25
3. The *Sac I* and *Hind III* DNA fragment containing CaMV 35S promoter and rbcS terminator isolated from pG35rbcX was blunt ended (using T4 polymerase) and ligated to the blunt ended *Apa I* and *Sal I* digested pMLJ1 to create pML35rbc.  
30 This vector (8.45 kb) has been designated as pPUH11.

- 20 -

4. A DNA fragment containing the full-length FRS-LOX cDNA (nucleotide 1 to 2871, See FIG. 2, SEQ ID NO:2) was isolated from clone 10-1#4-1 after digestion with *Pst*I, and *Xho*I and blunt-ended with T4 DNA Polymerase. This DNA fragment  
5 was cloned in both orientations in the blunt-ended pPUH11 at *Xho*I site to create pUSL2 (sense) and pUAL2 (antisense) vectors, respectively. Restriction mapping and Southern blotting with CaMV 35S, LOX and rbcS 3' probes and DNA  
10 sequencing were used to establish structure of pUSL2 and pUAL2.

## EXAMPLE FIVE

## Construction of pUEL300s/pUEL300A

1. A DNA fragment containing nucleotides 1 to 297 of FRS-LOX (FIG. 3, SEQ ID NO:3) was obtained after polymerase  
15 chain reaction using a LOX-specific primer (a 17-mer oligonucleotide with the sequence 5' GGGTGATGTCTGTAAGC3' corresponding to the nucleotides 297-281) and the T3 Primer (a 17-mer primer specific for the T3 promoter located 90 bp  
20 upstream of the FRS-LOX cDNA sequence in pBluescript). This DNA fragment (0.387 kb) was cloned into *Eco*RV T-tailed pBluescript KS to yield pKSRS17.

2. The LOX specific DNA fragment was obtained from pKSRS17  
25 after *Eco*RI digestion and cloned in both orientations into an *Eco*RI digested pPUH11 using T4 DNA ligase to create pUEL300S (sense) and pUEL300A (antisense) chimeric genes, respectively. Restriction mapping and Southern blotting with CaMV 35S, LOX and rbcS 3' probes, and DNA sequencing  
30 were used to establish structure of pUEL300S and pUEL300A.

## EXAMPLE SIX

Transfer of Vectors to *Agrobacterium*

The vectors pUSL2, pUAL2, pMLSL, pMLAL, pUEL300S and  
35 pUEL300A were mobilized into *Agrobacterium tumefaciens*

- 21 -

strain pGV3850 using standard tri-parental mating techniques with a helper plasmid pGJ23. Transformers were selected using appropriate antibiotics. Total DNA was isolated from selected strains, digested with several endonucleases, separated on agarose gels by electrophoresis, blotted to membrane and hybridized to various probes to confirm the presence of all parts of the respective chimeric gene in *Agrobacterium*.

#### EXAMPLE SEVEN

##### Creation of Transgenic Plants

Cotyledons from eight day old tomato plants were cut and placed on tobacco cell feeder layers for 24 hours before infecting with an *Agrobacterium* strain (as prepared according to Example 6) harboring plasmid containing a chimeric gene. After a 30 minute infection period, the cotyledons were placed back on the tobacco cell feeder layers and incubated for an additional 48 hours before transferring onto a tomato regeneration medium containing kanamycin and cefotaxime. Every two weeks the explants were subcultured into new regeneration media. Regenerated shoots were rooted using a tomato rooting medium. The rooted plants were removed from tissue culture and placed in a growth chamber in soil for 2 weeks. The plants were then moved to the greenhouse. The presence of the inserted DNA in transgenic plants was confirmed using DNA gel blotting of genomic DNA obtained from transformed (regenerated) tomato plants.

#### EXAMPLE EIGHT

##### Level of FRS-LOX mRNA and Protein in Plants Transformed with pMLSL

The levels of FRS-LOX mRNA and protein in ripening fruits from the primary transgenic tomato plants expressing pMLSL analyzed were about 50% of that wild type parental fruits. FRS-LOX mRNA and protein levels were determined

- 22 -

using RNA gel blotting and immuno blotting, respectively. Since seeds from these primary transgenic plants segregated in a normal Mendelian manner they are believed to be herterozygous for the introduced pMLSL construct.

5

#### EXAMPLE NINE

##### Effect of Reduced FRS-LOX on Ripening-Associated Fruit Firmness

The level of firmness of whole transgenic fruits transformed with the nucleotide sequence of SEQ ID NO:1 in sense orientation were determined by using a McCormick Fruit Tech (Yakima, WA) firmness pressure tester. This tester has a plunger tip and measures the penetration force necessary for the plunger tip to enter the fruit epidermis. In performing these tests, about a 1 cm square section of the waxy epidermis from each fruit was removed where the measurement was to be taken. A minimum of six fruits were analyzed and the mean and standard error were calculated. FIG. 6 shows the comparison of ripening-associated fruit firmness over time post breaker in PMSL-650-1 fruits (transgenic tomato transformed with the pMLSL vector) and wild type parental plants. Table 3 shows the firmness of fruits from segregating progeny of PMSL-650-1 with zero, one and two copies of the inserted pMLSL gene. These data clearly demonstrate that reduction of the FRS-LOX inhibits ripening-associated fruit softening.



- 23 -

Table 3. Firmness of Fruits from Transgenic Tomato Plant Containing 0, 1 and 2 copies of inserted pMLSL Gene.

5	Days After Breaker Stage	Fruits Firmness, lbs.		
		Copies of Inserted Gene		
		0	1	2
	7	8.85+/-0.96	9.51+/-0.67	15.51+/-1.94
	10	6.29+/-0.49	7.43+/-0.42	11.46+/-2.93

10 While the invention has been described in detail in  
the foregoing description, the same is considered as  
illustrative and not restrictive in character, it being  
understood that only the preferred embodiments have been  
shown and described and that all changes and modifications  
15 that come within the spirit of the invention are desired to  
be protected.

- 24 -

What is claimed is:

1. A method for making a transformed plant having improved fruit quality comprising:

5 inserting a foreign nucleotide sequence into DNA of a plant in a sense or antisense orientation so as to inhibit production of fruit ripening specific lipoxygenase in a fruit produced by the plant.

2. The method according to claim 1, wherein the plant is a tomato plant.

10 3. The method according to claim 1, wherein said inserting comprises:

providing a vector comprising the foreign nucleotide sequence; and

15 contacting the vector with a target cell of the plant such that the nucleotide sequence becomes incorporated into the target cell.

20 4. The method according to claim 1, wherein the nucleotide sequence has substantial similarity to all or a portion of the coding region of the plant's native fruit ripening specific lipoxygenase gene.

5. The method according to claim 1, wherein the nucleotide sequence has substantial similarity to all or a portion of the sequence of SEQ ID NO:4.

25 6. The method according to claim 1, wherein the nucleotide sequence has substantial similarity to the nucleotide sequence set forth in SEQ ID NO:1.

SUBSTITUTE SHEET (RULE 26)

- 25 -

7. The method according to claim 1, wherein the nucleotide sequence has substantial similarity to the nucleotide sequence set forth in SEQ ID NO:2.

5 8. The method according to claim 1, wherein the nucleotide sequence has substantial similarity to the nucleotide sequence set forth in SEQ ID NO:3.

9. A method for inhibiting lipoxygenase production in a plant comprising:

10 inserting a nucleotide sequence into DNA of the plant in the sense or antisense orientation so as to inhibit production of fruit ripening specific lipoxygenase in the fruit of the plant during ripening;

15 wherein the nucleotide sequence is substantially similar to all or a portion of the plant's native fruit ripening specific lipoxygenase.

20 10. A vector for inserting a nucleotide sequence into a fruit-bearing plant genome comprising a nucleotide sequence, in either a sense or an antisense orientation, having substantial similarity to all or a portion of a nucleotide sequence of a fruit ripening specific lipoxygenase native to the plant being transformed.

25 11. The vector according to claim 10 comprising a nucleotide sequence having substantial similarity to the nucleotide sequence of SEQ ID NO:1 in either a sense or an antisense orientation.

12. The vector according to claim 10 comprising a nucleotide sequence having substantial similarity to the nucleotide sequence of SEQ ID NO:2 in either a sense or an antisense orientation.

SUBSTITUTE SHEET (RULE 26)

- 26 -

13. The vector according to claim 10 comprising a nucleotide sequence having substantial similarity to the nucleotide sequence of SEQ ID NO:3 in either a sense or an antisense orientation.

5 14. A DNA construct selected from the group consisting of pMLSL, pMLAL, pUSL2, pUAL2, pUEL300S or pUEL300A.

15. A transgenic plant cell comprising a foreign nucleotide sequence in its genome in the sense or antisense orientation, the foreign nucleotide sequence being  
10 substantially similar to all or a portion of a fruit ripening specific lipoxygenase native to the plant being transformed.

16. A transgenic plant comprising a foreign nucleotide sequence in its genome in the sense or antisense  
15 orientation, the foreign nucleotide sequence being substantially similar to all or a portion of the plant's native fruit ripening specific lipoxygenase.

17. The transgenic plant of claim 16, the foreign nucleotide sequence being substantially similar to the nucleotide  
20 sequence of SEQ ID NO:1.

18. The transgenic plant of claim 16, the foreign nucleotide sequence being substantially similar to the nucleotide sequence of SEQ ID NO:2.

19. The transgenic plant of claim 16, the foreign nucleotide  
25 sequence being substantially similar to the nucleotide sequence of SEQ ID NO:3.

20. A transgenic fruit produced by the plant of claim 16.

**SUBSTITUTE SHEET (RULE 26)**

- 27 -

21. The transgenic fruit according to claim 20, wherein the foreign nucleotide sequence is substantially similar to a member selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO:1; the nucleotide sequence  
5 set forth in SEQ ID NO:2; or the nucleotide sequence set forth in SEQ ID NO:3.

22. The transgenic fruit according to claim 16 which is a tomato.

SUBSTITUTE SHEET (RULE 26)

## FIG. 1

SEQ ID NO:1

158 TTC AGTTGTTGAT GGCATTTCTG ATTTACTTGG CCAAAAAGTC  
201 TCTATCCAAT TGATAAGTGG TTCTGTAAAT TATGATGGTT TGGAAGGGAA  
251 ACTGAGCAAT CCAGCATACT TAGAGAGTTG GCTTACAGAC ATCACCCEAA  
301 TAACAGCAGG GGAATCAACT TTTAGTGTTA CATTTGACTG GGATCGTGAC  
351 GAGTTTGGAG TTCCAGGAGC ATTCATCATC AAGAATCTTC ATCTTAATGA  
401 GTTCTTTCTC AAGTCACTCA CACTCGAAGA TGTTCCCTAAT TATGGAAAAA  
451 TCCATTTTGT ATGCAATTCT TGGGTTTATC CTGCTTTTAG ATACAAGTCT  
501 GACCGCATT TCTTTGCCAA TCAGGCTTAT CTCCCAAGTG AAACACCACA  
551 ACCATTGCGA AAATACAGAG AAAATGAACT GGTAGCTTTG CGAGGAGATG  
601 GAACTGGAAA GCTTGAAGAA TGGGACAGGG TTTATGATTA TGCTTGCTAC  
651 AATGACTTGG GTGAACCAGA TAAGGGGGAA GAGTATGCTA GGCCTATCCT  
701 TGGAGGGTCC TCTGAGTACC CGTATCCTCG TAGAGGCAGG ACAGGCCGCG  
751 AACCAACCA AGCAGATCCT AATTGCGAGA GCAGGAACCC ATTGCCTATG  
801 AGCTTAGACA TATATGTCCC AAGGGACGAG CGATTGAGTC ATGTGAAGAA  
851 GTCAGACTTT TTGACGTCGT CCTTAAATC CTCTTTGCAA ACGCTTCTCC  
901 CTGCGTTTAA GGCTTTGTGC GATAACACGC CTAATGAGTT CAATAGCTTT  
951 GCGGATGTAC TTAATCTCTA TGAAGGAGGA ATCAAGTTGC CTGAAGGCCC  
1001 TTGGTTGAAA GCCATTACTG ATAACATTTT CTCAGAGATA CTAAGAGACA  
1051 TCCTTCAAAC GGATGGTCAA GGCCTACTTA AGTACCCAAC TCCTCAGGTT  
1101 ATTCAAGGCG ATAAACTGC ATGGAGGACG GATGAAGAAT TTGGGAGAGA  
1151 AATGTTGGCA GGATCCAATC CTGTCTTAAT CAGTAGACTC CAAGAATTTC  
1201 CTCCGAAGAG CAAGTTGGAT CCAACCATAT ATGGAAACCA AAACAGTACA  
1251 ATTACCACAG AACATGTACA GGATAAGTTG AATGGATTAA CAGTGAATGA  
1301 GGCAATCAAG AGTAACAGGT TATTCATATT GAACCACCAT GACATCGTGA  
1351 TGCCACTATT GAGGAAAATT AACATGTCAG CAAACACAAA AGCCTATGCC  
1401 TCAAGAACTC TGCTCTTCCT ACAAGATGAT AGAACTTTGA AGCCACTAGC  
1451 AATTGAACTA AGCTTGCCAC ATCCAGACGG AGATCAATTT GGTACTGTTA  
1501 GTAAAGTATA TACACCAGCT GACCAAGGTG TTGAAGGTTT TATCTGGCAG  
1551 TTTGCCAAAG CCTATGTAGC AGTGAATGAC ATGGGCATTG ATCAGCTCAT  
1601 TAGCCACTGG TTGAATACAC ACGCGGTGAT CGAACCATTG GTGATTGCAA  
1651 CAAATAGGCA TCTAAGTGTG CTTCATCCCA TTCATAAACT TCTTCATCCT  
1701 CATTTCCGTA ACACGATGAA CATAAATGCT TTAGCAAGAG AGACCTTGAC  
1751 CTATGATGGT GGTTTTGAGA CGTCTCTTTT TCCTGCCAAA TATTCCATGG  
1801 AAATGTCAGC AGCAGCTTAC AAAGATTGGG TTTTCCCTGA ACAAGCACTT  
1851 CCTGCTGATC TCCTCAAAAG AGGAGTGGCT GTTGAGGACT TGAGCTCCCC  
1901 ACATGGCATT CGTTTACTGA TTCTGGACTA TCCATATGCT GTTGATGGCT  
1951 TGGAAATTTG GGCAGCAATC AAAAGTTGGG TAACAGAATA TTGCAAGTTC  
2001 TATTACAAAT CTGACGAGAC AGTAGAGAAA GACACTGAAC TCCAAGCTTG  
2051 GTGGAAGGAG CTCCGCGAAG AAGGACATGG CGACAAGAAA GATGAGGCTT  
2101 GGTGGCCTAA ACTGCAAACT CGACAAGAGC TCAGAGATTG TTGCACCATC

SUBSTITUTE SHEET (RULE 26)

2151 ATTATATGGA TAGCTTCAGC ACTTCATGCA GCACTCCATT TTGGCTTATA  
2201 CTCTTACGCT GGTATCTCC CTAATCGCCC TACTTTAAGC TGTAATTTGA  
2251 TGCCAGAGCC AGGAAGTGTT GAGTATGAAG AGCTCAAGAC AAATCCAGAC  
2301 AAGGTATTCC TAAAAACATT TGTCCTCAG TTGCAATCAC TGCTTGAAAT  
2351 TTCCATCTTT GAGGTCTCGT CAAGGCATGC TTCAGATGAG GTTTACTTGG  
2401 GACAAAGGGA CTCAATTGAA TGGACAAAGG ATAAAGAACC ACTTGTAAGT  
2451 TTTGAGAGGT TTGGAAAGAT GCTAAGTGAT ATCGAGAATC GAATTATGAT  
2501 AATGAATAGT CATAAGAGTT GGAAGAACAG GTCAGGGCCT GTTAACGTTT  
2551 CATATACGTT GCTCTTTCCC ACAAGTGAAG AGGGACTCAC AGGCAAAG

## FIG. 2

SEQ ID NO:2

1 TTTTCTTAAT TAAAAAATAA ATATTTCTGT TTAAATAGTT AATCATGTCT  
51 TTGGGTGGAA TTGTGGATGC CATCCTTGGA AAAGATGATA GGCCAAAAGT  
101 GAAAGGAAGA GTGATTTTGA TGAAAAAATA TGTTCCTAGAC TTCATTAATA  
151 TAGGTGCTTC AGTTGTTGAT GGCATTTCTG ATTTACTTGG CCAAAAAGTC  
201 TCTATCCAAT TGATAAGTGG TTCTGTTAAT TATGATGGTT TGGAAGGGAA  
251 ACTGAGCAAT CCAGCATACT TAGAGAGTTG GCTTACAGAC ATCACCACAA  
301 TAACAGCAGG GGAATCAACT TTTAGTGTTA CATTTGACTG GGATCGTGAC  
351 GAGTTTGGAG TTCCAGGAGC ATTCATCATC AAGAATCTTC ATCTTAATGA  
401 GTTCTTTCTC AAGTCACTCA CACTCGAAGA TGTTCCTAAT TATGGAAAAA  
451 TCCATTTTGT ATGCAATTCT TGGGTTTATC CTGCTTTTAG ATACAAGTCT  
501 GACCGCATT TCTTTGCCAA TCAGGCTTAT CTCCAAGTG AACACCACA  
551 ACCATTGCCA AAATACAGAG AAAATGAACT GGTAGCTTTG CGAGGAGATG  
601 GAACTGGAAA GCTTGAAGAA TGGGACAGGG TTTATGATTA TGCTTGCTAC  
651 AATGACTTGG GTGAACCAGA TAAGGGGGAA GAGTATGCTA GGCCTATCCT  
701 TGGAGGGTCC TCTGAGTACC CGTATCCTCG TAGAGGCAGG ACAGGCCGCG  
751 AACCAACCAA AGCAGATCCT AATTGCGAGA GCAGGAACCC ATTGCCTATG  
801 AGCTTAGACA TATATGTCCC AAGGGACGAG CGATTTGGTC ATGTGAAGAA  
851 GTCAGACTTT TTGACGTCGT CCTTAAATC CTCTTTGCAA ACGCTTCTCC  
901 CTGCGTTTAA GGCTTTGTGC GATAACACGC CTAATGAGTT CAATAGCTTT  
951 GCGGATGTAC TTAATCTCTA TGAAGGAGGA ATCAAGTTGC CTGAAGGCCC  
1001 TTGGTTGAAA GCCATTACTG ATAACATTTT CTCAGAGATA CTAAAAGACA  
1051 TCCTTCAAAC GGATGGTCAA GGCCTACTTA AGTACCCAAC TCCTCAGGTT  
1101 ATTCAAGGCG ATAAACTGC ATGGAGGACG GATGAAGAAT TTGGGAGAGA  
1151 AATGTTGGCA GGATCCAATC CTGTCTTAAT CAGTAGACTC CAAGAATTTT  
1201 CTCCGAAGAG CAAGTTGGAT CCAACCATAT ATGGAAACCA AAACAGTACA  
1251 ATTACCACAG AACATGTACA GGATAAGTTG AATGGATTAA CAGTGAATGA  
1301 GGCAATCAAG AGTAACAGGT TATTCATATT GAACCACCAT GACATCGTGA  
1351 TGCCACTATT GAGGAAAATT AACATGTCAG CAAACACAAA AGCCTATGCC  
1401 TCAAGAACTC TGCTCTTCCT ACAAGATGAT AGAACTTTGA AGCCACTAGC  
1451 AATTGAACTA AGCTTGCCAC ATCCAGACGG AGATCAATTT GGTACTGTTA  
1501 GTAAAGTATA TACACCAGCT GACCAAGGTG TTGAAGGTTT TATCTGGCAG  
1551 TTTGCCAAAG CCTATGTAGC AGTGAATGAC ATGGGCATT C ATCAGCTCAT  
1601 TAGCCACTGG TTGAATACAC ACGCGGTGAT CGAACCATT GTGATTGCAA  
1651 CAAATAGGCA TCTAAGTGTG CTTTATCCCA TTCATAAACT TCTTCATCCT  
1701 CATTTCCGTA ACACGATGAA CATAAATGCT TTAGCAAGAG AGACCTTGAC  
1751 CTATGATGGT GGTTTTGAGA CGTCTCTTTT TCCTGCCAAA TATTCCATGG  
1801 AAATGTCAGC AGCAGCTTAC AAAGATTGGG TTTTCCCTGA ACAAGCACTT  
1851 CCTGCTGATC TCCTCAAAAG AGGAGTGGCT GTTGAGGACT TGAGCTCCCC  
1901 ACATGGCATT CGTTTACTGA TTCTGGACTA TCCATATGCT GTTGATGGCT  
1951 TGGAAATTTG GGCAGCAATC AAAAGTTGGG TAACAGAATA TTGCAAGTTC

SUBSTITUTE SHEET (RULE 26)



2001 TATTACAAAT CTGACGAGAC AGTAGAGAAA GACACTGAAC TCCAAGCTTG  
2051 GTGGAAGGAG CTCGCGAAG AAGGACATGG CGACAAGAAA GATGAGGCTT  
2101 GGTGGCCTAA ACTGCAAAC CTGACAAGAGC TCAGAGATTG TTGCACCATC  
2151 ATTATATGGA TAGCTTCAGC ACTTCATGCA GCACTCCATT TTGGCTTATA  
2201 CTCTTACGCT GGTTATCTCC CTAATCGCCC TACTTTAAGC TGTAATTTGA  
2251 TGCCAGAGCC AGGAAGTGTT GAGTATGAAG AGCTCAAGAC AAATCCAGAC  
2301 AAGGTATTCC TAAAAACATT TGTTCTCAG TTGCAATCAC TGCTTGAAAT  
2351 TTCCATCTTT GAGGTCTCGT CAAGGCATGC TTCAGATGAG GTTTACTTGG  
2401 GACAAAGGGA CTCAATTGAA TGGACAAAGG ATAAAGAACC ACTTG TAGCT  
2451 TTTGAGAGGT TTGGAAAGAT GCTAAGTGAT ATCGAGAATC GAATTATGAT  
2501 AATGAATAGT CATAAGAGTT GGAAGAACAG GTCAGGGCCT GTTAACGTTT  
2551 CATATACGTT GCTCTTTCCC ACAAGTGAAG AGGGACTCAC AGGCAAAGGA  
2601 ATTCCCAACA GTGTGTCTAT ATAGAACTTA TTATTCAATC AGTTTGTTGT  
2651 GCTTGTTGTTA CTTGTTATTC CCAACCAAAT AAACCTTTTG TTCAAATAA  
2701 AGAGTATTGT ATTGTATTGT CTTGTGTGTT GTGTTGTATT GTATTATATT  
2751 GTATAGTATT ATTGATTTAA ATACAATGTT TGTTGCACTT GTTCTTGTT  
2801 ATTCCCAACC AAATAAACTC TTTGTTCCAA ATAAAGCTGT AGTTGGTTTT  
2851 AAAAAAAAAA AAAAAAAAAA A

**FIG. 3**

SEQ ID NO:3

```
1   TTTTCTTAAT TAAAAAATAA ATATTTCTGT TTAAATAGTT AATCATGTCT
51  TTGGGTGGAA TTGTGGATGC CATCCTTGGA AAAGATGATA GGCCAAAAGT
101 GAAAGGAAGA GTGATTTTGA TGAAAAAATA TGTTCTAGAC TTCATTAATA
151 TAGGTGCTTC AGTTGTTGAT GGCATTTCTG ATTTACTTGG CCAAAAAGTC
201 TCTATCCAAT TGATAAGTGG TTCTGTTAAT TATGATGGTT TGGAAGGGAA
251 ACTGAGCAAT CCAGCATACT TAGAGAGTTG GCTTACAGAC ATCACCC
```

**SUBSTITUTE SHEET (RULE 26)**

6/9

**Fig. 4**

Nucleotide sequence of ripening-specific LOX  
cDNA used for chimeric gene constructions.

SEQ ID NO:4

```

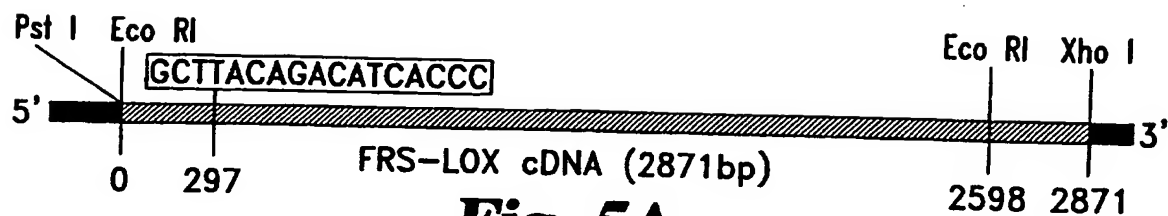
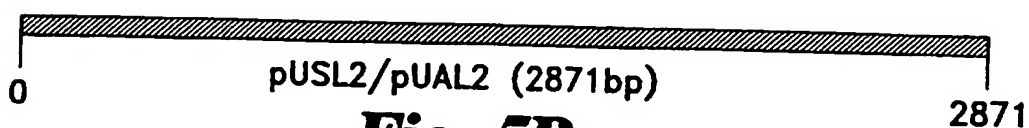
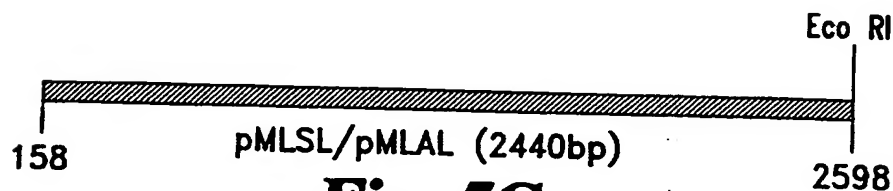
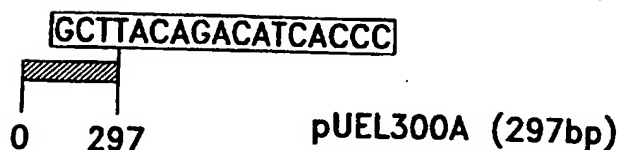
1   TTTTCTTAAT TAAAAAATAA ATATTTCTGT TTAAATAGTT AATCATGTCT
51  TTGGGTGGAA TTGTGGATGC CATCCTTGGA AAAGATGATA GGCCAAAAGT
101 GAAAGGAAGA GTGATTTTGA TGAAAAAATA TGTTCAGAC TTCATTAATA
151 TAGGTGCTTC AGTTGTTGAT GGCATTTCTG ATTTACTTGG CCAAAAAGTC
201 TCTATCCAAT TGATAAGTGG TTCTGTTAAT TATGATGGTT TGGAAGGGAA
251 ACTGAGCAAT CCAGCATACT TAGAGAGTTG GCTTACAGAC ATCACCCTCA
301 TAACAGCAGG GGAATCAACT TTTAGTGTTA CATTTGACTG GGATCGTGAC
351 GAGTTTGGAG TTCCAGGAGC ATTCATCATC AAGAATCTTC ATCTTAATGA
401 GTTCTTTCTC AAGTCACTCA CACTCGAAGA TGTTCCTAAT TATGGAAAAA
451 TCCATTTTGT ATGCAATTCT TGGGTTTATC CTGCTTTTAG ATACAAGTCT
501 GACCGCATT TCTTTGCCAA TCAGGCTTAT CTCCAAGTG AACACCACA
551 ACCATTGCGA AAATACAGAG AAAATGAACT GGTAGCTTTG CGAGGAGATG
601 GAACTGGAAA GCTTGAAGAA TGGGACAGGG TTTATGATTA TGCTTGCTAC
651 AATGACTTGG GTGAACCAGA TAAGGGGGAA GAGTATGCTA GGCCTATCCT
701 TGGAGGGTCC TCTGAGTACC CGTATCCTCG TAGAGGCAGG ACAGGCCGCG
751 AACCAACCAA AGCAGATCCT AATTGCGAGA GCAGGAACCC ATTGCCTATG
801 AGCTTAGACA TATATGTCCC AAGGGACGAG CGATTTGGTC ATGTGAAGAA
851 GTCAGACTTT TTGACGTCGT CCTTAAAATC CTCTTTGCAA ACGCTTCTCC
901 CTGCGTTTAA GGCTTTGTGC GATAACACGC CTAATGAGTT CAATAGCTTT
951 GCGGATGTAC TTAATCTCTA TGAAGGAGGA ATCAAGTTGC CTGAAGGCCC
1001 TTGGTTGAAA GCCATTACTG ATAACATTTT CTCAGAGATA CTAAAAGACA
1051 TCCTTCAAAC GGATGGTCAA GGCCTACTTA AGTACCCAAC TCCTCAGGTT
1101 ATTCAAGGCG ATAAAACCTGC ATGGAGGACG GATGAAGAAT TTGGGAGAGA
1151 AATGTTGGCA GGATCCAATC CTGTCTTAAT CAGTAGACTC CAAGAATTTT
1201 CTCCGAAGAG CAAGTTGGAT CCAACCATAT ATGGAAACCA AACAGTACA
1251 ATTACCACAG AACATGTACA GGATAAGTTG AATGGATTAA CAGTGAATGA
1301 GGCAATCAAG AGTAACAGGT TATTCATATT GAACCACCAT GACATCGTGA
1351 TGCCACTATT GAGGAAAATT AACATGTCAG CAAACACAAA AGCCTATGCC
1401 TCAAGAACTC TGCTCTTCCT ACAAGATGAT AGAACTTTGA AGCCACTAGC
1451 AATTGAACTA AGCTTGCCAC ATCCAGACGG AGATCAATTT GGTACTGTTA
1501 GTAAAGTATA TACACCAGCT GACCAAGGTG TTGAAGGTTT TATCTGGCAG
1551 TTTGCCAAAG CCTATGTAGC AGTGAATGAC ATGGGCATT C ATCAGCTCAT
1601 TAGCCACTGG TTGAATACAC ACGCGGTGAT CGAACCATT GTGATTGCAA
1651 CAAATAGGCA TCTAAGTGTG CTTTCATCCA TTCATAAACT TCTTCATCCT
1701 CATTTCCGTA ACACGATGAA CATAAATGCT TTAGCAAGAG AGACCTTGAC
1751 CTATGATGGT GGTTTTGAGA CGTCTCTTTT TCCTGCCAAA TATTCATGG
1801 AAATGTCAGC AGCAGCTTAC AAAGATTGGG TTTTCCCTGA ACAAGCACTT

```

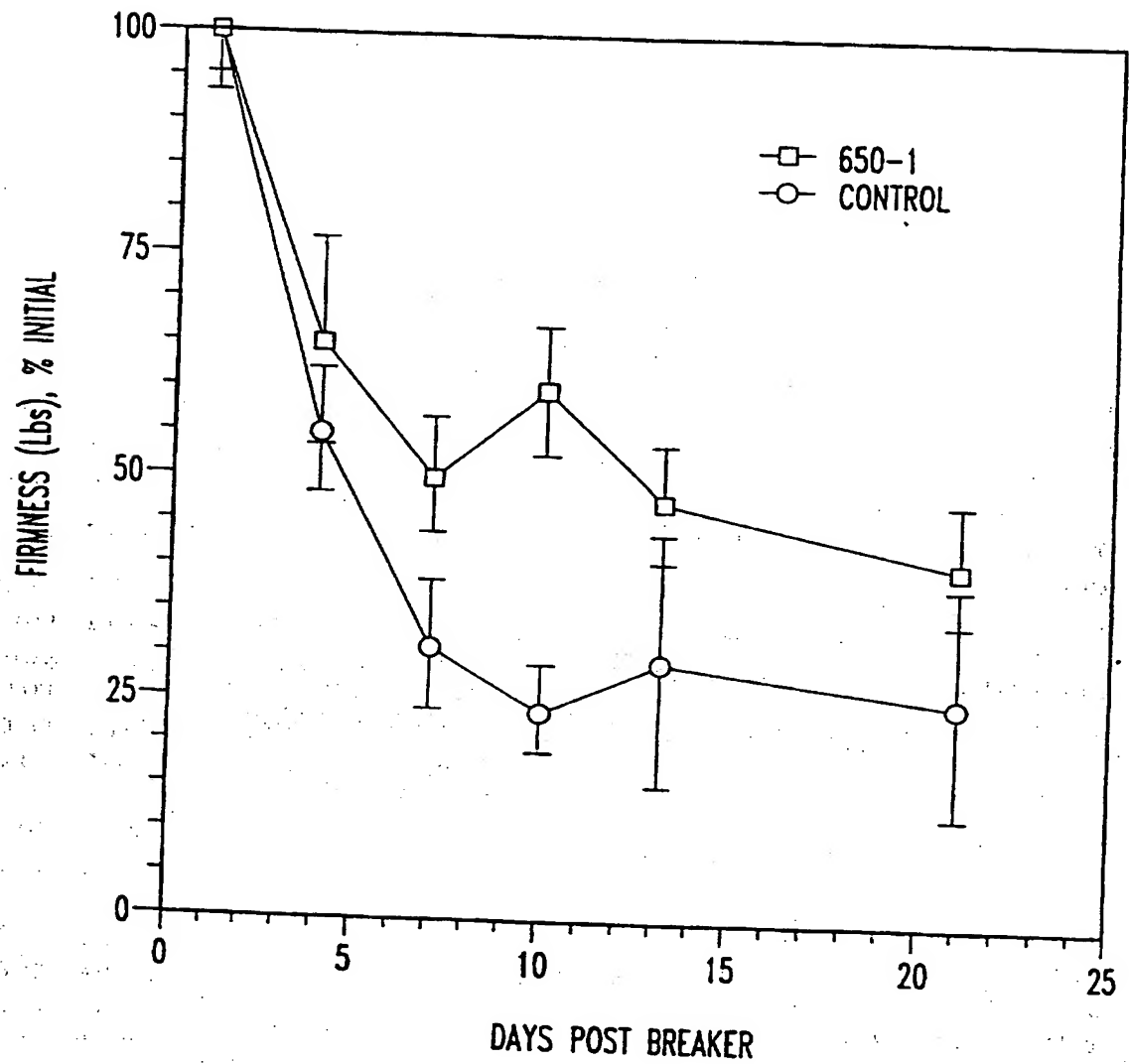
SUBSTITUTE SHEET (RULE 26)

1851 CCTGCTGATC TCCTCAAAAG AGGAGTGGCT GTTGAGGACT TGAGCTCCCC  
1901 ACATGGCATT CGTTTACTGA TTCTGGACTA TCCATATGCT GTTGATGGCT  
1951 TGGAAATTTG GGCAGCAATC AAAAGTTGGG TAACAGAATA TTGCAAGTTC  
2001 TATTACAAAT CTGACGAGAC AGTAGAGAAA GACACTGAAC TCCAAGCTTG  
2051 GTGGAAGGAG CTCCGCGAAG AAGGACATGG CGACAAGAAA GATGAGGCTT  
2101 GGTGGCCTAA ACTGCAAACT CGACAAGAGC TCAGAGATTG TTGCACCATC  
2151 ATTATATGGA TAGCTTCAGC ACTTCATGCA GCACTCCATT TTGGCTTATA  
2201 CTCTTACGCT GGTATCTCC CTAATCGCCC TACTTTAAGC TGTAATTTGA  
2251 TGCCAGAGCC AGGAAGTGTT GAGTATGAAG AGCTCAAGAC AAATCCAGAC  
2301 AAGGTATTCC TAAAAACATT TGTTCCTCAG TTGCAATCAC TGCTTGAAAT  
2351 TTCCATCTTT GAGGTCTCGT CAAGGCATGC TTCAGATGAG GTTTACTTGG  
2401 GACAAAGGGA CTCAATTGAA TGGACAAAGG ATAAAGAACC ACTTGTAGCT  
2451 TTTGAGAGGT TTGGAAAGAT GCTAAGTGAT ATCGAGAATC GAATTATGAT  
2501 AATGAATAGT CATAAGAGTT GGAAGAACAG GTCAGGGCCT GTTAACGTTT  
2551 CATATACGTT GCTCTTTCCC ACAAGTGAAG AGGGACTCAC AGGCAAAGGA  
2601 ATTCCCAACA GTGTGTCTAT ATAGAACTTA TTATTCAATC AGTTTGTTGT  
2651 GCTTGTGTTA CTTGTTATTC CCAACCAAT AAACCTTTTG TTCCAAATAA  
2701 AGAGTATTGT ATTGTATTGT CTTGTGTGTT GTGTTGTATT GTATTATATT  
2751 GTATAGTATT ATTGATTTAA ATACAATGTT TGTTGCACTT GTTTCTTGTT  
2801 ATTCCCAACC AAATAAACTC TTTGTTCCAA ATAAAGCTGT AGTTGGTTTT  
2851 AAAAAAAAAA AAAAAAAAAA A

SUBSTITUTE SHEET (RULE 26)

**Fig. 5A****Fig. 5B****Fig. 5C****Fig. 5D**

% FRUIT FIRMNESS (Lbs) MEASUREMENTS  
OF pMLSL 650-1 AND OHIO CONTROLS



**Fig. 6**

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/16387

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00, 15/05, 15/29, 15/64, 15/82; A01H 1/00, 4/00

US CL : 800/205, DIG 44; 435/172.3, 240.4, 320.1; 536/23.6, 24.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, DIG 44; 435/172.3, 240.4, 320.1; 536/23.6, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CABA, CAPLUS, MEDLINE, BIOSIS, GENBANK  
search terms: lipoxygenase, ripening, antisense, tomato

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,107,065 A (SHEWMAKER et al.) 21 April 1992, columns 1-5.	1-22
Y	US 5,254,800 A (BIRD et al.) 19 October 1993, columns 1-4.	1-22
Y	US 5,453,566 A (SHEWMAKER et al.) 26 September 1995, columns 1-10.	1-22
Y	FERRIE et al. The Cloning of Two Tomato Lipoxygenase Genes and Their Differential Expression during Fruit Ripening. Plant Physiol. 1994, Vol. 106, pages 109-118. especially pages 109-113.	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\*

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\*

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\*

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*A\*

document member of the same patent family

Date of the actual completion of the international search

09 DECEMBER 1996

Date of mailing of the international search report

21 JAN 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

THOMAS HAAS

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

**THIS PAGE BLANK (USPTO)**